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Abstract

An epileptic focus not resulting in generalized convulsions was induced by a microinjection of ferric chloride solution into the left anterior cortex of rats. The formation of the epileptic focus was confirmed by the appearance of bilateral spike and slow wave complexes as well as focal isolated spikes in electrocorticograms (ECoGs). The effect of glutamate on cyclic AMP accumulation was examined in incubated slices prepared from four quadrants of the epileptic cortex. In animals showing isolated spikes 8 to 10 days after the microinjection, the effect of glutamate on cyclic AMP accumulation was stimulatory. It was greatest in the left anterior quadrant which included the injection site, but only slight in the left and right posterior quadrants. In animals showing spike and slow wave complexes 30 to 60 days after the microinjection, the stimulatory effect of glutamate was also most pronounced in the left anterior quadrant. In the right anterior and the left posterior quadrants glutamate had almost no effect, while in the right posterior quadrant, glutamate was inhibitory.

KEYWORDS: rat cerebral cortex, iron-induced epileptic focus, cortical slices, glutamate, cyclic AMP

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DUAL EFFECTS OF GLUTAMATE ON CYCLIC AMP LEVELS IN SLICES OF RAT CEREBRAL CORTEX WITH AN IRON-INDUCED EPILEPTIC FOCUS

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Abstract. An epileptic focus not resulting in generalized convulsions was induced by a microinjection of ferric chloride solution into the left anterior cortex of rats. The formation of the epileptic focus was confirmed by the appearance of bilateral spike and slow wave complexes as well as focal isolated spikes in electrocorticograms (ECoGs). The effect of glutamate on cyclic AMP accumulation was examined in incubated slices prepared from four quadrants of the epileptic cortex. In animals showing isolated spikes 8 to 10 days after the microinjection, the effect of glutamate on cyclic AMP accumulation was stimulatory. It was greatest in the left anterior quadrant which included the injection site, but only slight in the left and right posterior quadrants. In animals showing spike and slow wave complexes 30 to 60 days after the microinjection, the stimulatory effect of glutamate was also most pronounced in the left anterior quadrant. In the right anterior and the left posterior quadrants glutamate had almost no effect, while in the right posterior quadrant, glutamate was inhibitory.

Key words : rat cerebral cortex, iron-induced epileptic focus, cortical slices, glutamate, cyclic AMP.

Willmore *et al.* (1-3) first reported that an intracortical application of a ferrous or ferric chloride solution in rats could induce an acute or chronic epileptic focus in a very high percentage of the cases. Histological examinations have shown that iron- or hemoglobin-induced epileptic foci can be considered to be a proper model of human posttraumatic epilepsy (2, 4). However, few reports are available on the biochemical processes involved in the development of the iron-induced focus. Recently, Rubin and Willmore (5) suggested that intracortically applied iron compounds formed superoxide radicals which caused peroxidation of membranous components of cortical tissue and that these processes resulted in regional degeneration of cortical cells and finally in the induction of epileptic discharges.

Studies of acute epileptic foci induced by topical penicillin application (6) or

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local freezing (7) imply that fluctuation in the levels of cortical cyclic nucleotides is an important factor in the development of focal epilepsy.

In relation to these implications, we speculated that some persistent change in the cyclic AMP-generating system may occur in rat cerebral cortex with an iron-induced epileptic focus. Glutamate has been shown to elicit the accumulation of cyclic AMP in slices prepared from rat cerebral cortex (8, 9) and to be related to experimentally induced epilepsy (10-13). In the present study, to evaluate the formation of cyclic AMP in different areas of the epileptic cortex, we measured the cyclic AMP content after incubation of cortical slices in the presence or absence of glutamate.

MATERIALS AND METHODS

Male Wistar rats weighing 220 to 280 g were used. The surgical procedures were essentially the same as those of Willmore *et al.* (1, 2) and Tada *et al.* (14). Under ether anesthesia a small trephine hole was made in the cranial bone over the left sensorimotor cortex at a point 1.5 mm rostral and 3.5 mm lateral to the bregma. Through the hole the needle of a microsyringe was inserted, and its tip was set at a depth of 1.5 mm from the dorsal surface of the bone. For the animals in the experimental group, 5 μl of 100 mM sterilized $FeCl_3$ solution was injected into the cortex slowly (*ca.* 1 μl/min). For the animals in the control group, the same amount of saline was injected in the same way. For recording electrocorticograms (ECoGs), four stainless steel screws were implanted in the cranial bone. The anterior ones were situated 3 mm rostral and 3 mm lateral to the bregma and the posterior ones 4 mm caudal and 4 mm lateral to the bregma on both sides. ECoG recordings of epileptic discharges started one week after the intracortical injection of $FeCl_3$ solution and were repeated two or three times a week for about 10 weeks.

Experimental rats were sacrificed by decapitation 8 to 10 days (early stage) or 30 to 60 days (late stage) after the intracortical injection of $FeCl_3$ solution with confirmation of the persistence of epileptic discharges by ECoG. Control animals were sacrificed comparable times after the injection of saline. After decapitation the cerebrum was rapidly excised and immersed in an ice-cold Krebs-Ringer bicarbonate buffer saturated with a mixture of 95 % O_2 and 5 % CO_2. The buffer consisted of 118 mM NaCl, 4.7mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.7 mM glucose. The cerebral cortex was dissected into four parts of nearly equal volume, *i.e.*, the left anterior, right anterior, left posterior, and right posterior quadrants. Each quadrant of the cortex was cross-chopped to 500 μm thickness with a McIlwain tissue chopper. The chopped slices from each quadrant were gently stirred in the buffer to homogeneity, and then the suspension was separated into two nearly equal portions. Each portion (4-6 mg protein) was preincubated in 5 ml of the Krebs-Ringer bicarbonate buffer for 20 min at 37 ℃. After the preincubation, the buffer of one portion of the suspension was replaced with 5 ml of fresh buffer containing 10 mM L-glutamate, while the buffer of the other was replaced with 5 ml of fresh buffer without L-glutamate. Then, the suspensions were further incubated at 37 ℃. The suspension was constantly aerated with the gas mixture noted above throughout both the preincubation and incubation. Ten minutes later, the incubation was followed by decanting of the suspension buffer and adding 2.2 ml of cold 7 % trichloroacetic acid to the slices. After homogenization of the resultant mixture in an ice-bath, [^{3}H]cyclic AMP (0.05 pmol, about 3000 dpm, for

recovery check) and 0.2 ml of 1 N HCl were added to 2.0 ml of the homogenate. Precipitated protein in the homogenate was removed by centrifugation (2000 × *g*, 15 min) at 4 ℃. The supernatant was vigorously shaken with water-saturated ether ; then the ether layer was discarded by aspiration. This procedure was repeated four times. The remaining solution containing cyclic AMP was applied onto a column (0.7 × 3 cm) of Dowex 50W-X8 (200-400 mesh, H^+-form) (15). Cyclic AMP was eluted with 5.5 ml of distilled water after the column was washed with 3 ml of 0.1 N HCl and then with 0.5 ml of distilled water. The eluate was lyophilized and used for the cyclic AMP assay.

The lyophilized residues were dissolved in 0.4 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM EDTA. For the recovery check, 0.1 ml of each dissolved sample was used. Cyclic AMP contained in 0.05 ml of the samples was assayed by a cyclic AMP assay kit (Radiochemical Centre, Amersham, U.K.). The assay procedure was based on the method of Gilman (16). Cyclic AMP contents in cortical slices were expressed as pmol per mg protein. The protein content of the homogenate was determined by the method of Lowry *et al.* (17) using bovine serum albumin as the standard.

Statistical significance of changes in cyclic AMP contents was evaluated by Student's *t*-test.

RESULTS

ECoG and behavior. Electrographic seizure activity appeared in nearly 100 % of the experimental animals. The activity persisted for more than 10 weeks after the intracortical injection of $FeCl_3$ solution. Fig. 1 shows one ECoG record obtained 8 to 10 days after the injcetion. Isolated spikes can be seen in the lead of the left anterior cortex, which often developed into bilateral spike and slow wave complexes about three weeks after the injection. Fig. 2 shows such an ECoG record of bilateral spike and slow wave complexes obtained 35 days after the injection. Behavioral features of the seizure activity, that is, the peripheral manifestations of the focus, were restricted to intermittent twitching of the face or neck muscles at the most. Saline-injected animals had normal ECoGs.

Cyclic AMP levels in incubated cortical slices. When the experimental animals were sacrificed at the early stage, cyclic AMP levels in the slices from each quadrant of the cortex ranged from 35.8 to 40.4 pmol/mg protein after incubation in

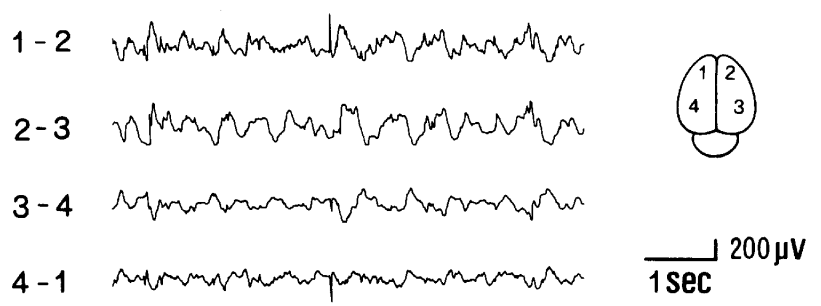

Fig. 1. Isolated spikes in an ECoG recorded 9 days after injection of $FeCl_3$ solution.

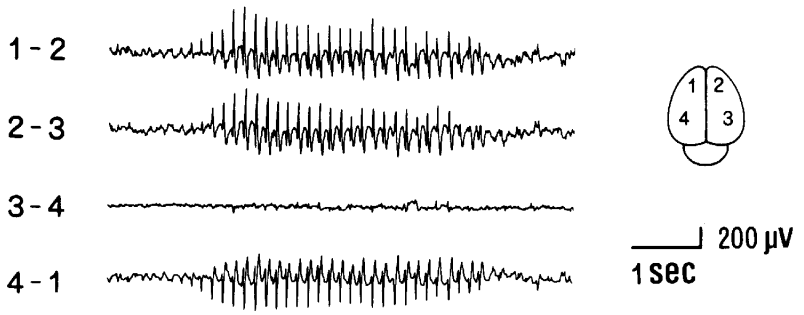

Fig. 2. Bilateral spike and slow wave complexes in an ECoG recorded 35 days after injection of $FeCl_3$ solution.

TABLE 1. EFFECT OF GLUTAMATE ON CYCLIC AMP LEVELS IN INCUBATED SLICES FROM FOUR CORTICAL AREAS OF RATS AT THE EARLY STAGE

Treatment and cortical area	Cyclic AMP (pmol/mg protein) after incubation in	
	Buffer alone	Buffer with glutamate
$FeCl_3$ solution injection		
Left anterior	35.8± 4.4	75.2± 12.4[b]
Right anterior	39.4± 4.9	62.2± 8.1[a]
Left posterior	37.6± 5.8	49.2± 4.4
Right posterior	40.4± 4.5	42.4± 6.8[e]
Saline injection		
Left anterior	24.8± 2.8	48.4± 3.7[d]
Right anterior	27.4± 2.6	52.8± 4.7[d]
Left posterior	26.2± 3.2	48.8± 4.9[d]
Right posterior	28.2± 3.1	53.4± 6.5[c]

Animals were sacrificed 8 to 10 days after the injection. The concentration of glutamate in the incubation medium was 10 mM. Each value represents the mean±S.E.M. of five experiments.

[a] $p < 0.05$; [b] $p < 0.02$; [c] $p < 0.01$; [d] $p < 0.005$, compared with the corresponding value in the absence of glutamate.

[e] $p < 0.05$, compared with the value of the left anterior area under the same incubation conditions.

the absence of glutamate (Table 1). There was no obvious regional difference. When the cortical slices from the same animal group were incubated in glutamate-containing buffer, the cyclic AMP levels varied among the cortical quadrants. In the left anterior quadrant, the cyclic AMP level was highest and significantly different from the corresponding value in the absence of glutamate ($p < 0.02$). In the right anterior quadrant, the cyclic AMP level was also significantly higher

than the corresponding value in the absence of glutamate ($p<0.05$). The increase of cyclic AMP level was slight in the left posterior quadrant. In the right posterior quadrant, the addition of glutamate seemed to produce no clear increase in the cyclic AMP level, which was significantly lower than that in the left anterior quadrant ($p<0.05$).

In control animals sacrificed at the early stage, the cyclic AMP levels in cortical slices which were incubated without glutamate ranged from 24.8 to 28.2 pmol/mg protein (Table 1). There was no regional difference. After incubation with glutamate, the cyclic AMP levels increased and were roughly twofold (48.4-53.4 pmol/mg protein) the corresponding values of all four quadrants, and again there was no regional difference.

In experimental animals sacrificed at the late stage, the cyclic AMP levels of slices from each quadrant of the cortex were about 40 pmol/mg protein after incubation without glutamate, and there was no regional difference (Table 2). A clear regional difference in cyclic AMP levels was observed when the slices were incubated with glutamate. The cyclic AMP level was highest in the left anterior quadrant (73.6 pmol/mg protein) and was significantly different from the value in the absence of glutamate ($p<0.01$). The effect of glutamate was hardly detected in the right anterior and the left posterior quadrants. In these quadrants, the cyclic AMP levels after incubation with glutamate were significantly lower

TABLE 2. EFFECT OF GLUTAMATE ON CYCLIC AMP LEVELS IN INCUBATED SLICES FROM FOUR CORTICAL AREAS OF RATS AT THE LATE STAGE

Treatment and cortical area	Cyclic AMP (pmol/mg protein) after incubation in	
	Buffer alone	Buffer with glutamate
$FeCl_3$ solution injection		
Left anterior	39.4± 3.2	73.6± 9.3[b]
Right anterior	41.6± 4.4	39.6± 8.2[d]
Left posterior	37.6± 5.7	43.2± 8.7[d]
Right posterior	43.2± 5.0	23.2± 4.4[a, e]
Saline injection		
Left anterior	31.8± 4.5	60.4± 7.2[b]
Right anterior	29.2± 2.3	55.0± 5.4[c]
Left posterior	33.2± 4.9	61.8± 7.7[a]
Right posterior	31.4± 3.8	56.6± 6.8[a]

Animals were sacrificed 30 to 60 days after the injection. The concentration of glutamate in the incubation medium was 10 mM. Each value represents the mean± S.E.M. of five experiments.

[a] $p<0.02$; [b] $p<0.01$; [c] $p<0.005$, compared with the corresponding value in the absence of glutamate.

[d] $p<0.05$; [e] $p<0.005$, compared with the value of the left anterior area under the same incubation conditions.

than the cyclic AMP level in the left anterior quadrant under the same incubation conditions ($p < 0.05$). Moreover, the cyclic AMP level in the slices from the right posterior quadrant after incubation with glutamate was significantly lower than the corresponding value in the absence of glutamate ($p < 0.02$), and also significantly lower than the value in slices from the left anterior quadrant after incubation with glutamate ($p < 0.005$).

In control animals sacrificed at the late stage, the cyclic AMP levels were about 30 pmol/mg protein after the incubation without glutamate and about 60 pmol/mg protein after incubation with glutamate (Table 2). There was no regional difference in the cyclic AMP levels in the presence or absence of glutamate.

DISCUSSION

It has been reported that the accumulation of cyclic AMP is elicited in brain slices by incubation with putative neurotransmitters or neuromodulators (18-20). Biogenic amines and adenosine are effective in eliciting the accumulation of cyclic AMP in brain tissue of rats (21-24). Glutamate is known to be an excitant of the nervous system (25, 26) and also to elicit the accumulation of cyclic AMP in brain slices of guinea pigs (8, 27, 28) and mice (29). However, evidence that, in rat cerebral cortex, glutamate has a stimulatory effect on cyclic AMP accumulation is to be found in few reports (8, 9). All of these reports indicate that the occurrence and the extent of cyclic AMP accumulation depend upon the animal species, the central nervous structures from which slices were prepared or the test substances.

In the present work, a small but definite increase of cyclic AMP content was detected in the cerebral cortical slices of rats by incubation with glutamate. The results clearly showed that there is a regional difference in the responsiveness of the cyclic AMP-generating system to glutamate in slices of rat cerebral cortex with an iron-induced epileptic focus. This suggests that modification of cell membrane characteristics in various cortical regions progresses with a different time course and scale according to their relation to the epileptic focus.

An increased release of glutamate has been reported to occur in connection with cobalt-induced epileptic foci (12, 13). If such an increase also occurs with iron-induced epileptic foci, the present results may be a reflection of the regional differences in glutamate levels of the epileptic cortex. Two types of adenosine receptors have been found in cultured brain cells : the A1 receptor, which mediates the inhibition, and the A2 receptor, which mediates the stimulation of cyclic AMP accumulation (30, 31). Analogous to the effects of adenosine, the dual effects of glutamate on the cyclic AMP level in the present study might be due to the presence of two types of glutamate receptors in rat cerebral cortex. However, on the other hand, the effect of glutamate on cyclic AMP levels may be indirect since the release of adenosine or adenosine and other unknown factors

involved in cyclic AMP generation has been reported in glutamate-treated tissue (32-34) and cell-free preparations (35). Taken together with results of previous studies, the present findings suggest that alterations in extracellular levels of adenosine or glutamate are associated with the process of the iron-induced epileptic focus.

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